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Research paper

Rapid and sensitive detection of cytokines using functionalized gold nanoparticle-based immuno-PCR, comparison with immuno-PCR and ELISA

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ABSTRACT

Reliable and simple methods are required for detection of low concentrations of cytokines and some other proteins in complex biological fluids. This is especially important when monitoring the immune responses under various physiological and pathophysiological conditions *in vivo* or following production of these compounds in *in vitro* systems. Cytokines and other immunologically active molecules are being predominantly detected by enzyme-linked immunosorbent assays (ELISA) and newly also by immuno-polymerase chain reactions (iPCR). New simplified variants of iPCR have recently been described where antibodies are connected with multiple DNA templates through gold nanoparticles (Au-NPs) to form a new class of detection reagents. In this study we compared functionalized Au-NP-based iPCR (Nano-iPCR) with standard ELISA and iPCR for the detection of interleukin (IL)-3 and stem cell factor (SCF). The same immunoreagents (IL-3- and SCF-specific polyclonal antibodies and their biotinylated forms) were used throughout the assays. The obtained data indicate that both Nano-iPCR and iPCR are superior in sensitivity and detection range than ELISA. Furthermore, Nano-iPCR is easier to perform than the other two methods. Nano-iPCR was used for monitoring changes in concentration of free SCF during growth of mast cells in SCF-conditioned media. The results show that growing cultures gradually reduce the amount of SCF in supernatant to 25% after 5 days. The combined data indicate that Nano-iPCR assays may be preferable for rapid detection of low concentrations of cytokines in complex biological fluids.

1. Introduction

Cytokines are small signaling protein molecules that are produced by cells of diverse embryonic origin and serve as important mediators of the immune system. Abnormal activities of several cytokines, including interleukin (IL)-3, have been reported in schizophrenia (Chen and Kendler, 2008), and elevated levels of stem cell factor (SCF) have been detected in asthmatic patients (Lei et al., 2008). Sensitive, simple and robust methods are required for diagnostic purposes to determine low concentrations of cytokines in complex biological fluids. They are needed for monitoring immune responses *in vivo* as well as for rapid analysis of the quality of conditioned media used in culturing cytokine-dependent cells. Growth of mouse bone marrow-derived mast cells (BMMCs) *in vitro*, is promoted by two cytokines, IL-3 and SCF (Tsuji et al., 1991). Concentrations of these and other cytokines are being determined by several methods, such as bioassays employing cytokine-dependent freshly isolated cells or cell lines (Chen et al., 1993). Although very useful, these assays are time consuming and inaccurate.

Abbreviations: IL, interleukin; SCF, stem cell factor; BMMC, bone marrow-derived mast cell; ELISA, enzyme-linked immunosorbent assay; RT, reverse-transcription; PCR, polymerase chain reaction; iPCR, immuno-PCR; Au-NP, gold nanoparticle; Nano-iPCR, functionalized Au-NP-based iPCR; r, recombinant; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate buffer solution; BSA, bovine serum albumin; TPBS, PBS containing 0.05% Tween 20; HRP, horseradish peroxidase; OPD, o-phenylenediamine; C_q, quantification cycle.

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Widespread methods used for detection of cytokines are enzyme-linked immunosorbent assays (ELISA) utilizing antibodies specific for the target proteins (Silman and Katchalski, 1966; Engvall and Perlmann, 1971). However, the sensitivity of these assays is not always sufficient. It has been shown that the amount of cytokine produced by cells correlates with the expression of cytokine-specific mRNA; reverse-transcription (RT) polymerase chain reactions (PCR) have therefore also been widely used. Although RT-PCRs are fast, sensitive and simple methods to detect the expression levels of cytokine genes, the results do not always agree with those of bioassays and ELISA, and do not allow exact determination of cytokine concentrations. Other assays have therefore been explored. In 1992 a new technique was described, combining molecular specificity of antibodies with the amplification power and sensitivity of PCR (Sano et al., 1992). In this technique, called immuno-PCR (iPCR), the antibody used for detection of target compound is combined with the DNA which serves as a template in PCR. Different strategies have been applied for linking antibodies with DNA templates, like streptavidin bridge combined with biotinylated antibody and biotinylated DNA template, or chemically conjugated antibody-DNA complexes (Lind and Kubista, 2005; Niemeyer et al., 2007). The amount of DNA amplified during PCR corresponds to the amount of target structure recognized by the antibody, and can be detected by electrophoresis (Zhou et al., 1993) or by ELISA, utilizing digoxigenin- or biotin-labeled PCR products (Niemeyer et al., 1997; Smrž and Dráber, 2003). Later, immunodetection was combined with real-time PCR and used for quantification of vascular endothelial grow factor (Sims et al., 2000). The method was further modified in such a way that both protein detection and real-time PCR were performed in the same well of the TopYield strip (Niemeyer et al., 2007). Furthermore, a gold nanoparticle (Au-NP)-based bio-bar code assay for ultrasensitive detection of proteins has been developed. The assay utilizes Au-NPs functionalized with both thiolated single-strand DNA oligonucleotide and an antibody to the target antigen (Nam et al., 2003, 2004; Georganopoulou et al., 2005). Finally, PCR assays based on antibody- and oligonucleotide-functionalized Au-NPs were used for detection of Hantaan virus nucleocapsid protein (Chen et al., 2009) and respiratory syncytial viruses (Perez et al., 2011). Although the assays showed high sensitivity for virus detection, they required two sets of wells (for immunodetection and PCR) and therefore were not suitable for high throughput screening and were fraught with high risk of contamination.

Here we tested the suitability of functionalized Au-NPs-based iPCR (Nano-iPCR) for detection of low concentrations of cytokines in cell culture supernatants, and changes in cytokine concentration in aging cultures of BMMCs. We defined the conditions for simplified detection of cytokines by Nano-iPCR, and compared the performance of assays based on antibodies anchored either directly on the plastic surface or through extravidin. The assays were carried out in PCR polypropylene wells or wells of TopYield polycarbonate strips which allow more efficient binding of antibodies. We further compared Nano-iPCR with iPCR and ELISA; outline of the assays is shown in Fig. 1. For these comparisons we utilized identical immunoreagents in all assays. The data indicate that Nano-iPCR offers a sensitive, rapid and robust assay for detection of low concentrations of cytokines in complex biological fluids. Advantages and drawbacks of different assays are discussed.

2. Materials and methods

2.1. Materials

Rabbit anti-murine IL-3 and rabbit anti-murine SCF polyclonal antibodies and their biotinylated forms, recombinant (r) murine IL-3 and rSCF were all obtained from PeproTech (London, UK). Colloidal Au-NPs (30 nm), containing approximately 2×10^{11} Au-NPs/ml, were obtained from BBInternational (Cardiff, UK). Au-NPs (5 nm) conjugated with goat anti-rabbit IgG were bought from Jackson ImmunoResearch Lab., Inc. (West Grove, PA, USA). 5′thiol-modified oligonucleotide primer (Pri)1 [5′-(5ThioMC6-D//iSp18)CCTTGAACCTGTGCCATTTGAATATATTAAGACTATACGCGGGAACA-3′] where iSp18 is an 18-atom hexa-ethyleneglycol spacer connecting the thiol reactive group and the DNA sequence, Pri2 (5′-CCTTGAACCTGTGCCATTTG-3′) and Pri3 (5′-GTCCCTCCATCTTCCTACTGTTCCACATGTTCCCGCGTATAGTCTT-3′) were obtained from IDT, (Coralville, IA, USA). Biotinylated forward primer 5B-HRM1-F (5′-CTCATCACCACGCTCCATTA-3′) and its non-biotinylated form (HRM1-F) and reverse primer HRM1-R (5′-TCTTCCACCTCCATGGTGTC-3′) were obtained from Generi Biotech (Hradec Králove, Czech Republic). SYTO-9 and geneticin (G418) were obtained from Invitrogen (Eugene, OR, USA). Glutaraldehyde was bought from Fluka, Chemie GmbH (Buchs, Switzerland). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cells

BMMCs were isolated from C57BL/6 mice according to the previously reported protocol (Volná et al., 2004). All mice were maintained and used in accordance with the Institute of Molecular Genetics guidelines. The cells were seeded in complete culture medium RPMI-1640 containing 10% heat inactivated (56 °C, 30 min) fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and further supplemented with fresh rSCF (15 ng/ml) and 10% culture supernatant of confluent D11 cells as a source of IL-3 (Cao et al., 2007). BMMCs were cultured at 37 °C in an atmosphere of 5% CO_2 in air. D11 cells were grown adherent in tissue culture flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated FBS, antibiotics and 0.3 mg/ml of geneticin. The cells were detached from the flasks by incubation for 5–10 min at room temperature with 0.05% trypsin in phosphate buffered saline (PBS; 10 mM phosphate, pH 7.4, 150 mM NaCl) supplemented with 0.02% EDTA. After centrifugation at 280 ×*g* for 5 min, cells were resuspended in DMEM-10% FCS with geneticin. After 3 days of culturing, the medium was aspirated and fresh DMEM medium without geneticin was added. Cells were cultured for additional week. Supernatant containing IL-3 was then collected, centrifuged at 5500 ×*g* for 15 min, filtered through a 0.22 μm membrane and stored in aliquotes at −20 °C. For determination of SCF, supernatant from cultured BMMCs was collected daily for 5 days.

2.3. Au-NPs functionalized with DNA and antibodies

Functionalized Au-NPs were prepared as previously described (Hill and Mirkin, 2006) with some modifications. One milliliter of 30 nm Au-NPs solution was incubated for 30 min at room temperature with 4 μg of polyclonal antibody (anti-IL-3 or anti-SCF) under gentle shaking. Ten microliters of 10% Tween 20 was then added, followed after 5 min by 50 μl of 2 M NaCl in 10 mM PBS (Hurst et al., 2008). The particles were then modified with 5′-thiolated oligonucleotide (final concentration 4 nmol/ml) under gentle shaking at 4 °C. After overnight incubation the samples were further salted by adding 50 μl aliquotes of 2 M NaCl in 10 mM PBS in 4 subsequent steps, each for 1 h. Functionalized Au-NPs were stabilized by adding 20 μl of 10% bovine serum albumin (BSA), followed by gentle shaking for 30 min at room temperature. Unbound oligonucleotides were removed by three times centrifugation (9.300 ×*g* for 10 min) through a discontinuous glycerol gradient in 2 ml Eppendorf tubes. The gradient consisted of 800 μl PBS containing 30% glycerol (w/v) and 1% BSA (w/v), overlaid with 1 ml of functionalized Au-NPs. The pellet was finally resuspended in 1 ml of PBS containing 1% BSA, 0.05% Tween 20, 20% glycerol and 0.02% NaN_3. In some experiments Au-NPs were functionalized with BSA instead of antibody. Absorption spectra were recorded with a UV-1601 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with software package UVProbe (Shimadzu) and quartz cells (200 μl) with 10 mm path length.

2.4. Electron microscopy

Nickel electron microscopy grids coated with pioloform were glow discharged and coated with poly-L-lysine. Au-NPs functionalized with antibodies or with BSA were allowed to settle on the coated grids. After 10 min, the grids were washed in PBS and free protein-binding sites were blocked for 15 min with 0.1% BSA in PBS. Grids with bound Au-NPs were then incubated with the secondary antibody (goat anti-rabbit IgG, conjugated with 5 nm Au-NPs), diluted 1:10 in PBS-0.1% BSA. After 30 min the grids were washed three times for 5 min each with PBS. The grids were fixed in 2.5% glutaraldehyde in PBS for 10 min. Finally, samples were washed twice with MilliQ water (Millipore, Billerica, MA, USA) for 1 min, air-dried and examined with a JEOL JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan) operating at 60 kV.

2.5. Nano-iPCR

For detection of cytokines by Nano-iPCR, two methods were used differing in the mode of anchoring the antibodies to plastic surface. In Nano-iPCR I biotinylated antibody was attached to immobilized extravidin, whereas in Nano-iPCR II the antibody was directly bound to the plate.

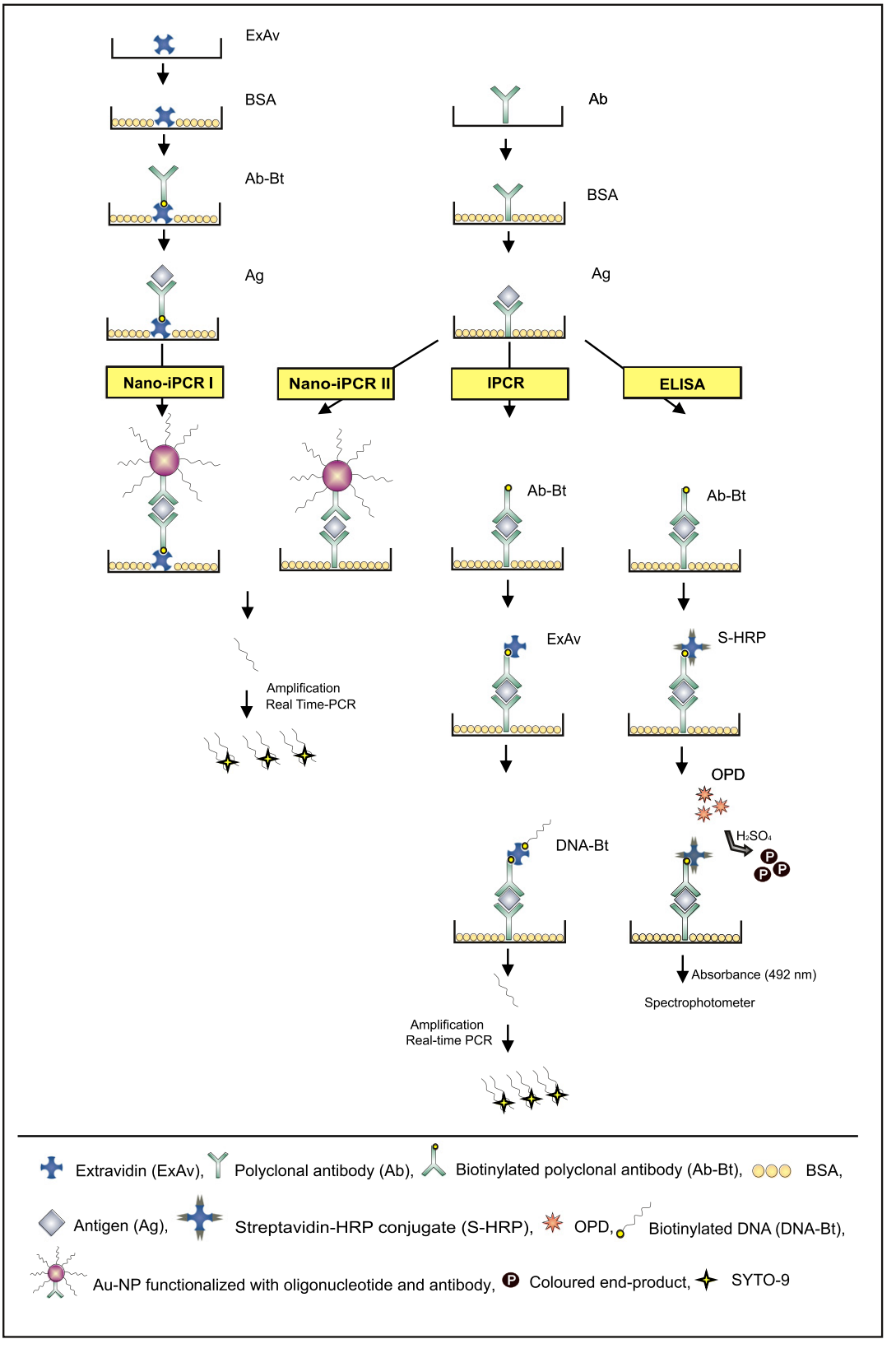
2.5.1. Nano-iPCR I

One hundred microliter aliquotes of extravidin (1–2 μg/ml in 100 mM borate buffer, pH 9.5) were added into each well of real-time 96-well plate or real-time tube strip (Eppendorf, Hamburg, Germany) and incubated for 1 h at 37 °C. After adsorption of the protein, the wells were washed with PBS containing 0.05% Tween 20 (TPBS) and the remaining binding sites were blocked by 2 h incubation at 37 °C with TPBS supplemented with 2% BSA. The wells were then washed three times with 200 μl of TPBS, followed by addition of 100 μl biotinylated anti-SCF or anti-IL-3 antibody (1 μg/ml in TPBS-1% BSA). After incubation for 1 h at 37 °C, unbound antibody was rinsed out and 100 μl sample aliquotes were probed for the presence of SCF or IL-3. The samples were incubated overnight at 4 °C, and after washing four times with TPBS, 100 μl of Au-NPs armed with thiolated DNA oligonucleotide and anti-SCF or anti-IL-3 (diluted 1/10,000 in TPBS-1% BSA) were applied into each well. The wells were incubated for 1 h at 37 °C, washed 4 times with 200 μl TPBS followed by double washing with MilliQ water. Next, aliquotes of 25 μl of master mix solution containing 75 mM Tris–HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 200 μM dTTP, Taq DNA polymerase (25 U/ml), 2 μM SYTO-9 and 60 nM oligonucleotide primers Pri2 and Pri3 were dispensed into each well. Plates were sealed with Light cycler 480 sealing foil (Roche, Mannheim, Germany) and PCR strips with Masterclear cap strips (Eppendorf). The amount of template DNA bound to antigen-anchored functionalized Au-NPs was evaluated by real-time PCR using Realplex^4 Mastercycler (Eppendorf, Hamburg, Germany) with the following cycling conditions: 1 min at 94 °C, followed by 40 cycles of 20 s at 94 °C, 20 s at 53 °C and 20 s at 72 °C. The control without template DNA was used for PCR mix in every run to check for contamination.

2.5.2. Nano-iPCR II

Twenty-five microliter aliquotes of capture antibody (5 μg/ml anti-IL-3 or anti-SCF; Peprotech) in 100 mM borate buffer (pH 9.5) were distributed into each well of TopYield strips (NUNC, Roskilde, Denmark). After 1 h incubation at 37 °C and overnight incubation at 4 °C each well was washed four times with 200 μl of TPBS, and free binding sites were blocked with TPBS-2% BSA for 2 h at 37 °C. Each well was washed four times with TPBS, followed by addition of 25 μl of the tested sample containing IL-3 or SCF and incubation for 1 h at 37 °C. Other steps were the same as in Nano-iPCR I. Cycling conditions were as follows: 2 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C and 60 s at 72 °C.

Fig. 1. Schematic illustration of the immunoassays used in this study. In Nano-iPCR I method, wells of real-time 96-well plate or real-time tube strips were coated with extravidin (ExAv) to which biotinylated antigen-specific antibody (Ab-Bt) was anchored. BSA was used to block free binding sites. Antigen (Ag) was then added and bound to the immobilized antibody. The antibody anchored antigen was detected by Au-NPs functionalized by antigen-specific antibody and thiolated oligonucleotide which served as a template in subsequent real-time PCR with a set of template-specific primers. DNA amplicons were detected by fluorometry using DNA binding dye SYTO-9. In Nano-iPCR II method, the antibody (Ab) was bound directly to the TopYield strip wells followed by blocking free protein-binding sites with BSA; all other steps were the same as in Nano-iPCR I. In iPCR and ELISA, the Ag-specific antibody was adsorbed directly onto the surface of the well, followed by BSA block. Antigen was then added and detected by biotinylated antibody. In iPCR, extravidin was supplied to form a bridge between the biotinylated antibody and biotinylated DNA template (DNA-Bt) added during the next step. Template DNA was then amplified by real-time PCR (using a set of template-specific primers), and the amplicons were detected by means of SYTO-9 DNA dye. In ELISA, biotinylated antibody immobilized on Ag reacted with streptavidin-HRP conjugate (S-HRP). The enzymatic activity was detected using a colorimetric reaction with OPD as a substrate. The colored end-product (P) was quantified by spectrophotometery.


ExAv
BSA
Ab-Bt
Ag
Nano-iPCR I
Nano-iPCR II
IPCR
ELISA
Ab
BSA
Ag
Ab-Bt
Ab-Bt
Amplification
Real Time-PCR
ExAv
S-HRP
OPD
H_2SO_4
DNA-Bt
Absorbance (492 nm)
Spectrophotometer
Amplification
Real-time PCR
Extravidin (ExAv),
Polyclonal antibody (Ab),
Biotinylated polyclonal antibody (Ab-Bt),
BSA,
Antigen (Ag),
Streptavidin-HRP conjugate (S-HRP),
OPD,
Biotinylated DNA (DNA-Bt),
Au-NP functionalized with oligonucleotide and antibody,
Coloured end-product,
SYTO-9

2.6. iPCR

The method was performed as previously described (Niemeyer et al., 2007) with some modifications. Biotinylated DNA template (221 bps) was obtained by PCR using biotinylated forward primer 5B-HRM1-F (200 nM), reverse primer HRM1-R (800 nM) and amplified template DNA (0.1 ng; GenBank accession no. M14752). The following cycling conditions were used: 2 min at 95 °C, followed by 30 cycles of 15 s at 95 °C, 30 s at 58 °C and 20 s at 72 °C. Each well of the TopYield strip contained 25 µl polyclonal antibody specific for IL-3 or SCF (5 µg/ml, in 100 mM borate buffer pH 9.5). The wells were incubated for 1 h at 37 °C and overnight at 4 °C, followed by washing four times with 200 µl of TPBS. Free binding sites were blocked by incubation with TPBS-2% BSA. After 2 h at 37 °C, the wells were again washed four times and overlaid with 25 µl of tested samples containing various concentrations of IL-3 or SCF. The wells were further incubated for 1 h 37 °C and then washed 4 times with TPBS. Subsequently, 25 µl aliquotes of biotinylated antibody specific for IL-3 or SCF (1 µg/ml in TPBS-1% BSA) were dispensed and the samples were incubated for 1 h at 37 °C. After washing four times, the wells were covered with 25 µl of extravidin (0.1 µg/ml) in TPBS-1% BSA, incubated for 1 h at 37 °C and washed as before. After this step, biotinylated DNA template (25 µl; 0.27 pg/ml) was added and the strips were incubated for 1 h at 37 °C. Next, the wells were washed four times with TPBS and twice with MilliQ water. The amount of biotinylated DNA template immobilized in individual wells was quantified by real-time PCR using master mix supplemented with HRM1-F and HRM1-R primer set (200 nM each). The following cycling conditions were used: 2 min at 95 °C as an initial denaturation step and 40 cycles consisting of 15 s at 95 °C, 60 s at 60 °C and elongation for 60 s at 72 °C.

2.7. ELISA

ELISA was performed as previously described (Engvall and Perlmann, 1971) with some modifications. Wells of the TopYield strips were coated with Ag-specific polyclonal antibody, blocked with TPBS-2% BSA and then mixed with antigen as described above for iPCR. The wells were then washed three times with TPBS, followed by addition of 100 µl biotinylated antibody (1 µg/ml, in TPBS-1% BSA), incubation for 1 h at 37 °C and washing three times with TPBS. One hundred microliters of streptavidin-horseradish peroxidase (HRP) conjugate (0.1 µg/ml) was then added. After incubation for 1 h at 37 °C the wells were washed three times with TPBS. Finally, 100 µl PBS containing o-phenylenediamine (OPD; 0.5 mg/ml) and H_2O_2 (0.015%) was dispensed into each well. After 10 min at 37 °C, the reaction was stopped by adding 100 µl of H_2SO_4 (4 M). The absorbance was determined at 492 nm using Infinite M200 plate reader (TECAN, Männedorf, Switzerland).

2.8. Data analysis

For calibration curves, absorbance or quantification cycle (C_q) values were plotted against SCF or IL-3 concentrations using a four-parameter logistic regression model function (variable slope) within GrafPad Prism 5 (GraphPad Software, La Jolla, CA, USA). For calculation of IL-3 or SCF concentrations in the tested samples, the same mathematical model was used, using MasterPlex ReaderFit software (Hitachi Solutions America, Ltd, MiraiBio Group, South San Francisco, CA, USA).

3. Results

3.1. Characterization of functionalized Au-NPs

Au-NPs functionalized with thiolated oligonucleotides and antibodies were initially characterized by two methods. The presence of antibodies bound to 30 nm Au-NPs was verified by means of secondary anti-immunoglobulin-specific antibodies conjugated to 5 nm Au-NPs. Formation of rosettes of 30 nm Au-NPs surrounded by 5 nm-Au-NPs detectable by electron microscopy was taken as an evidence of the presence of antibodies on 30 nm Au-NPs. As shown in Fig. 2A, all 30 nm Au-NPs formed rosettes with 5 nm particles. The binding was specific as indicated by the absence of rosettes in samples containing 30 nm Au-NPs covered with BSA instead of antibodies (Fig. 2B) or with thiolated oligonucleotides alone (not shown). A typical distribution pattern of 30 nm Au-NPs associated with 1–7 gold 5 nm particles is shown in Fig. 2C. It should be noted that the number of 5 nm particles bound to 30 nm Au-NPs is underestimated because a fraction of 5 nm particles is overshadowed by the dense bodies of 30 nm particles.

Concentrations and physical properties of functionalized Au-NPs were characterized by means of UV–vis spectrophotometry (Fig. 2D). Unconjugated 30 nm Au-NPs showed an absorption peak at 525 nm. This changed to higher wavelength values by 5–10 nm if the particles were functionalized by antibodies and oligonucleotides. Absorption maxima at values >535 nm were indicative of suboptimal performance of the particles in Nano-iPCR. The actual values of the 525–535 nm peak and calculated extinction coefficient [$\varepsilon_{528\ nm} = 3.7 \times 10^9\ cm^{-1}\ M^{-1}$ (Jin et al., 2003)] made it possible to determine the number of particles present in the sample.

The number of single-stranded oligonucleotides bound to 30 nm Au-NPs was evaluated by a modified real-time PCR-based method (Kim et al., 2006) where DNA binding dye SYTO-9 was used instead of a fluorescence probe. Au-NPs with bound thiolated Pri1 oligonucleotide were directly diluted into SYTO-9-containing PCR master mix supplemented with primers (Pri2 and Pri3), and analyzed by real-time PCR (Fig. 3A). Linearity of the data and regression coefficients close to 1 indicated that the presence of 30 nm Au-NPs did not interfere with the assay and therefore was not necessary to dissociate oligonucleotide template from Au-NPs before the PCR. Similar good linearity and reasonable regression coefficients were observed in assays containing a defined amount of free Pri1 oligonucleotide (Fig. 3B). Based on the results of such assays and estimated number of gold particles in stock solutions of functionalized Au-NPs it was possible to calculate the number of oligonucleotides per one oligonucleotide- and antibody-functionalized particle as 83 ± 26 (mean ± S.D.; $n = 5$). The $1/10^4$ dilution of functionalized Au-NPs in Nano-PCR assays corresponds to approximately 1.4 pmol/l of Pri1 oligonucleotide.

3.2. Optimization of the Nano-iPCR

The sensitivity of immunoassays is limited by background signal caused by nonspecific binding of assay components (primary and secondary antibodies, antigen, extravidin, biotinylated

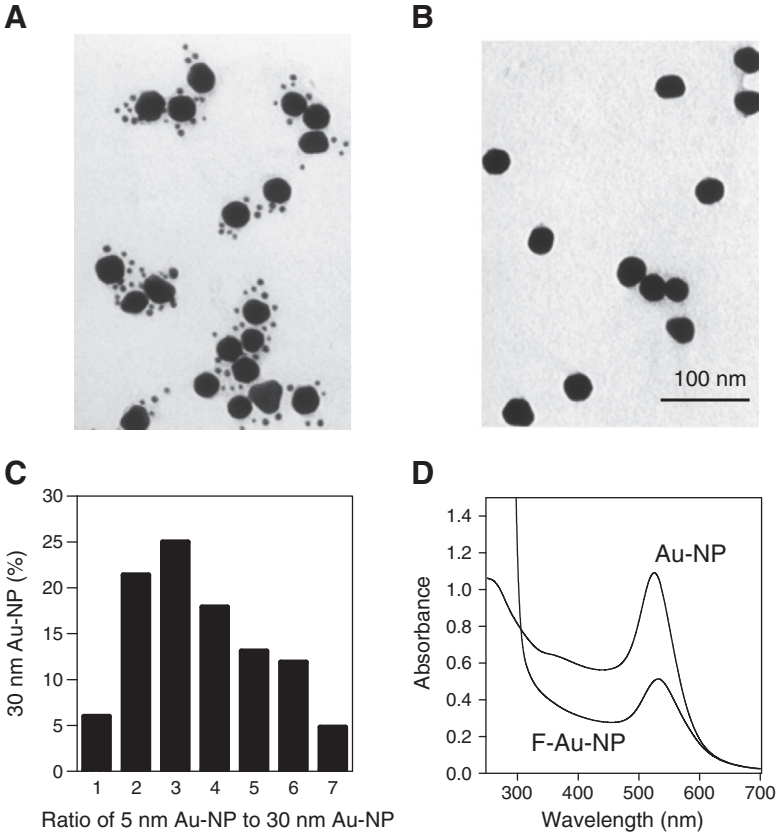


Fig. 2. Characterization of antibody-functionalized Au-NPs. (A, B) Electron microscopy visualization of polyclonal antibody bound to Au-NPs. (A) 30 nm Au-NPs were conjugated with polyclonal antibody followed by an exposure to secondary 5 nm Au-NP labeled with anti-IgG. After washing the particles were observed by electron microscopy. (B) Control 30 nm Au-NPs were conjugated with BSA, followed by an exposure to 5 nm Au-NP-labeled anti-IgG antibody. (C) Distribution of 5 nm Au-NPs (armed with anti-rabbit IgG) bound to 30 nm Au-NPs functionalized with polyclonal rabbit antibody. In total, 84 particles of 30 nm size were evaluated. (D) UV–vis spectrophotometry of control (Au-NP) and oligonucleotide- and antibody-functionalized particles (F-Au-NP).

DNA templates and/or functionalized Au-NPs) to both each other and plastic surfaces of the wells. In pilot experiments we therefore tried to define the optimal conditions for iPCR. We compared the performance of two buffers (PBS or HEPES) supplemented with several blocking agents at different concentrations (2–5% BSA, 2% ovalbumin or 2% casein) and two different detergents at various concentrations (0.01–0.2% Tween 20 or 0.1–2% Pluronic F68). A series of optimization experiments showed that the most effective agents for blocking and washing were TPBS-2% BSA and TPBS, respectively.

In initial experiments with TopYield strips we noticed, in accordance with previous studies (Barletta et al., 2004, 2005;

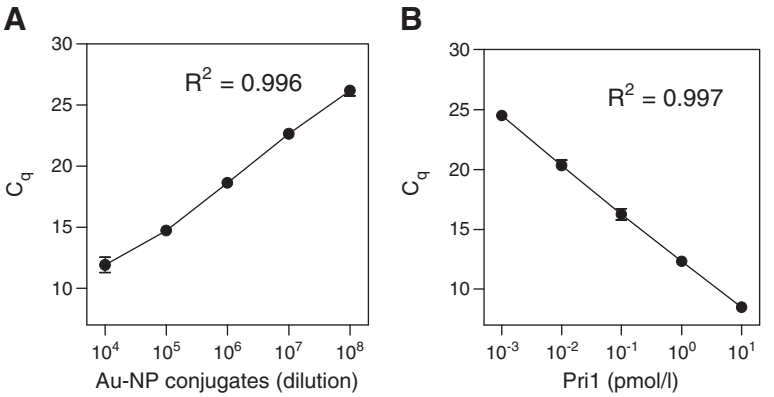


Fig. 3. Characterization of oligonucleotide-functionalized Au-NPs. (A) Real-time PCR performance of various dilutions of Au-NPs functionalized with Pri1 oligonucleotide. (B) Real-time PCR detection of various concentrations of oligonucleotide Pri1 alone. Correlation coefficients (R), means and S.D. were calculated from 3 independent experiments performed in triplicates.

Barletta, 2006) that there is a relatively high variability in the results and poor sensitivity. One possible cause was inferior heat transfer in wells of the TopYield strips during PCR. This was solved by extending the synthesis phase of the PCR cycles. Another problem was that larger surface area caused higher evaporation of PCR mixes, even though the wells were sealed with light cycler 480 sealing foil. We therefore added 5 µl of mineral oil into each well before they were sealed. Mineral oil prevented evaporation and improved the performance of detection of various concentrations of rIL-3 (Fig. 4) and rSCF (not shown).

3.3. Comparison of various immunoassays

To compare various immunoassays for detecting cytokines, we tested the performance of Nano-iPCR I and II, iPCR and ELISA for detection of rIL-3 and rSCF at various concentrations. For IL-3, polypropylene wells of the 96-well PCR plate (Eppendorf) were coated with extravidin, followed by anti-IL-3 polyclonal antibody (Nano-iPCR I). Alternatively, wells of TopYield strips (NUNC) were coated directly with anti-IL-3 antibody (Nano-iPCR II, iPCR and ELISA). Next, free binding sites were blocked with TPBS-2% BSA and rIL-3 at various concentrations was added. After incubation, unbound IL-3 was removed by washing with TPBS. Further course of the procedures differed depending on the method used (see Section 2 and Fig. 1). Analysis of data obtained showed that Nano-iPCR I (Fig. 5A) exhibited clear concentration-dependent differences in the range of 0.01–100 ng/ml of IL-3 with C_q values from ~35 (at 100 ng/ml) to ~46 (at 0.01 ng/ml). These relatively high values probably reflect low protein binding capacity of PCR polypropylene wells. Nano-iPCR II (Fig. 5C) performed in polycarbonate TopYiled strips showed lower C_q values in the range from ~19 (at 100 ng/ml) to ~32 (at 0.01 ng/ml). With iPCR (Fig. 5E), the dose–response curve was similar to that of Nano-iPCR II assay, except for even lower C_q values, from ~15 (at 100 ng/ml) to ~24 (at 0.01 ng/ml). This was in part caused by lower C_q values in negative controls (without IL-3) in iPCR compared to Nano-iPCR, and could be related to higher nonspecific binding of the biotinylated template used for iPCR. In contrast to Nano-iPCR and iPCR, ELISA assay (Fig. 5G) was less sensitive and the range of IL-3 concentrations detectable by the assay was narrower (between 0.1 and 10 ng/ml).

Similar data were obtained when various assays were used for detection of rSCF. Thus, Nano-iPCR I (Fig. 5B), compared to Nano-iPCR II (Fig. 5D) and iPCR (Fig. 5F), was characterized by relatively high C_q values (including negative controls without rSCF) and higher sensitivity at low concentration of rSCF. ELISA assay (Fig. 5H) was again less sensitive, and also the range of rSCF concentrations detectable by the assay was reduced (0.1–10 ng/ml). The data indicate that Nano-iPCRs and iPCRs are superior in sensitivity and exhibit broader range of detectable concentrations than ELISA.

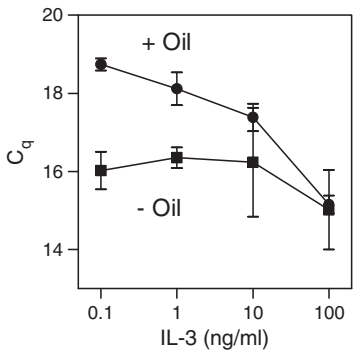


Fig. 4. The effect of mineral oil overlay on iPCR detection of IL-3 in wells of TopYield strips. Various concentrations of IL-3 were detected by iPCR in wells of TopYield strips overlaid (+) or not (−) with 5 µl of light mineral oil and sealed with real-time PCR transparent foil. Means and S.D. were calculated from 3 independent experiments performed in triplicates.

3.4. Cytokine detection in cell culture supernatants

To prove the convenience of Nano-iPCR we attempted to determine changes in the amount of rSCF during growth of BMMCs in RPMI-1640 medium supplemented with 10% FCS and SCF. Cell-free samples from cell cultures were collected at 24 h intervals for 5 days. In parallel, samples were also collected from cultures containing medium alone to determine the rate of degradation of SCF during incubated at 37 °C. Data in Fig. 6A show that growing cells gradually deplete the amount of rSCF. At the beginning of the experiment, samples contained SCF at concentration 15 ng/ml, but after 5 days, only about 3.7 ng SCF/ml remained (75% reduction). Similar data were obtained with ELISA. Degradation of rSCF in cell-free culture media had little effect, since the slight decrease there (9.5%) was only observed after 24 h of incubation and then remained constant. Binding of rSCF to the plasma membrane receptor (c-kit) and its internalization and subsequent degradation seems to be main cause of the observed depletion of rSCF from culture medium.

Next, we compared the efficiencies of various immunoassays for detection of IL-3 released into culture supernatant by growing D11 fibroblasts. Concentration of IL-3 was determined by Nano-iPCR II, iPCR and ELISA. Data presented in Fig. 6B indicate that D11 supernatant diluted 1:16 with TPBS-1% BSA contained 21.6 ± 2.0 ng/ml IL-3 (mean ± S.D.; $n = 3$), corresponding to 346 ± 32 ng/ml of undiluted supernatant. Higher dilutions of the supernatant had no effect on calculated concentration of IL-3. Similar data were obtained with iPCR (359 ± 90 ng/ml) or ELISA (384 ± 58 ng/ml).

4. Discussion

We compared three different immunoassays (Nano-iPCR, iPCR and ELISA) for detection of low concentrations of cytokines. Nano-iPCR was used in two formats differing in the mode how the antigen-specific antibodies were anchored to the reaction wells. In Nano-iPCR I, biotinylated antibody was anchored to immobilized extravidin, whereas in Nano-iPCR II the antibody was bound directly to the plastic surface. Both modifications used Au-NPs functionalized with single-stranded oligonucleotides and polyclonal antibodies specific for the cytokine in question. The assays gave reasonable concentration-dependent C_q values, although Nano-iPCR II showed higher nonspecific binding reflected by lower C_q values even in the absence of antigen. The components of the critical importance and limiting factors in all immunoassays are the antibodies, since they can differ in specificity and affinity for the target antigen, as well as nonspecific binding to the solid phase (McKie et al., 2002; Lind and Kubista, 2005; Niemeyer et al., 2007). It is therefore essential

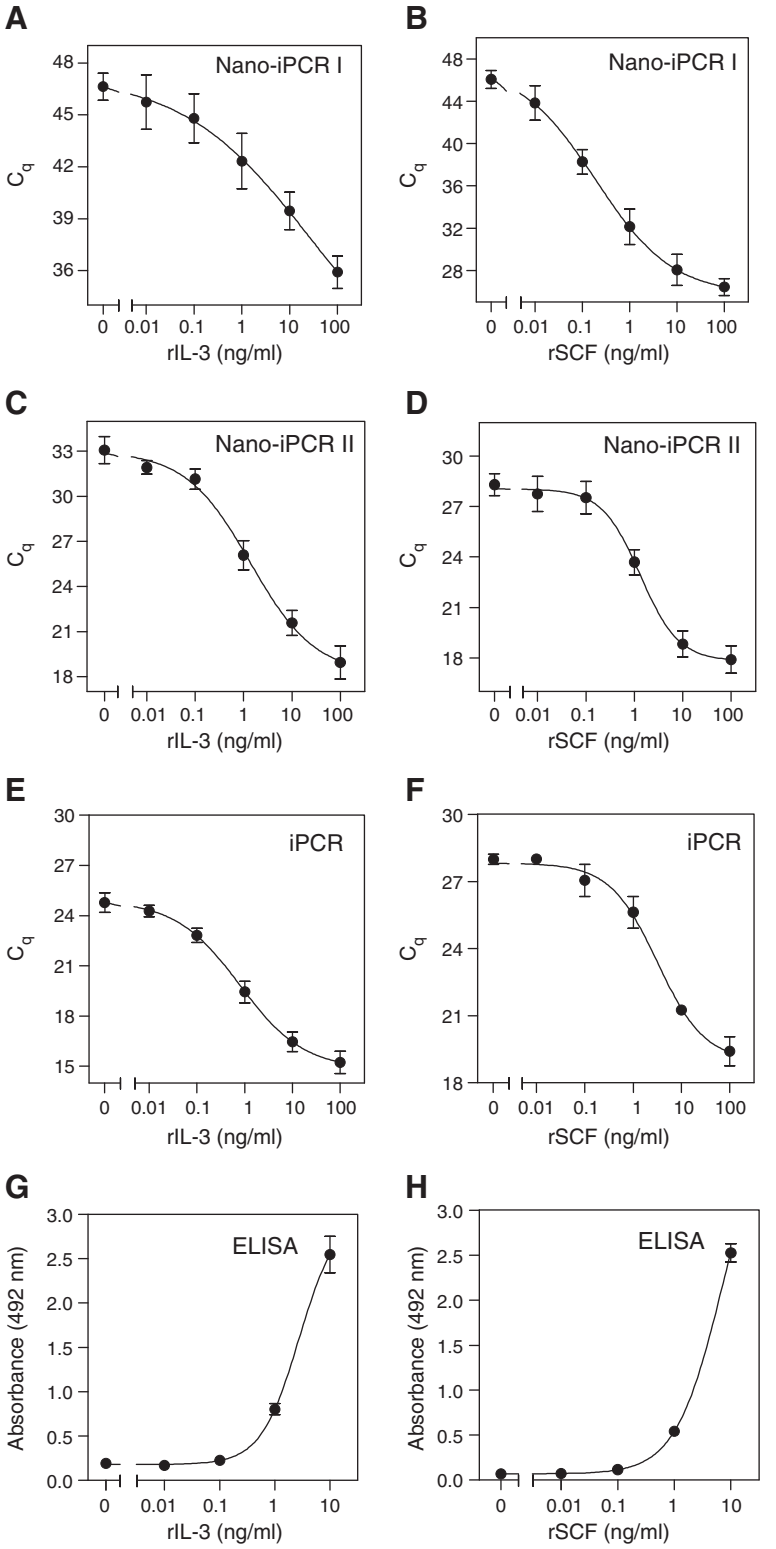


Fig. 5. Detection of various concentrations of rIL-3 and rSCF in particular immunoassays. Nano-iPCR I (A and B), Nano-iPCR II (C and D), iPCR (E and F) and ELISA (G and H) were used for detection of various concentrations of rIL-3 (A, C, E, G) or rSCF (B, D, F, H) in TPBS-1% BSA. Results are means ± S.D. from 3 to 6 independent experiment performed in triplicates or duplicates.

that in all the assays we employed the same sets of antibodies specific for IL-3 or SCF. The observed differences are thus attributable to the characteristics of the assays rather than antibodies.

The particular assays differed in a number of features which are summarized in Table 1. First, Nano-iPCR assays utilize functionalized Au-NPs. Production of such particles possessing both antibody and single-stranded oligonucleotides is more time consuming than preparation of biotinylated antibodies for ELISA and iPCR, or biotinylated double stranded oligonucleotides for iPCR. On the other hand, preparation of Au-NP conjugates is substantially easier than direct conjugation of antibodies with oligonucleotide templates (Lind and Kubista, 2005). As shown in this study, binding of the antibody to Au-NPs can be quantified by electron microscopy. The analysis proved that almost all Au-NPs bound several antibody molecules. The number of oligonucleotides bound to a particle was determined by real-time PCR using functionalized Au-NPs diluted directly into PCR mixes. Interestingly, even though each functionalized Au-NP possessed in average 80 oligonucleotides, performance of Nano-iPCR was comparable to the detection range of iPCR. This can be related to a higher background reflected in lower C_q values in iPCR calibration curves, including negative controls.

Second, an important parameter of immunoassays is the type of wells or tubes in which the assays are performed. An extensive array of various tubes, strips and plates fitting to different real-time PCR cyclers is available for PCR. However, these tubes and wells are often made of polypropylene and therefore exhibit a relatively low protein-binding capacity. At present, only the TopYield polycarbonate strips have antibody binding capacity comparable to polystyrene strips or plates widely used for ELISA, and have a shape compatible with heating blocks of various PCR cyclers. Our initial experiments showed that real-time PCR performance of TopYield strips was poor even in cyclers with heated lid. This was however improved by changing the cycling conditions and covering PCR master mixes with mineral oil. This obviously reduced evaporation from relatively large surface area of TopYield wells.

Third, both Nano-iPCR and iPCR detected the antigen with higher sensitivity than ELISA. This reflects the ability of PCR to amplify even a very small number of template DNA molecules. Initial studies indeed demonstrated a dramatic enhancement (approximately five orders of magnitude) in detection sensitivity when iPCR was used instead of ELISA (Sano et al., 1992). However, these assays were performed under optimal conditions where antigen (BSA) was directly immobilized to wells and a potent monoclonal antibody specific for BSA was available. When the antigen is present in a complex protein mix, such as in serum-containing culture medium or in crude body fluids, and analyzed in a sandwich assay, Nano-iPCR and iPCR usually detect the antigen with 1–3 orders higher sensitivity than ELISA (Adler et al., 2003; Lind and Kubista, 2005; Chen et al., 2009; Perez et al., 2011). In a study aimed at detecting mumps-specific IgG in serum samples, sensitivity of the iPCR did not exceed that of conventional ELISA. It should be kept in mind that Nano-iPCR and iPCR assays are substantially less sensitive for quantification of antigenic molecules when compared to real-time PCR for quantification of DNA templates. This is attributable to high specificity of PCR and zero amplification in the absence of DNA template.

Fourth, another important factor is the variability of the assays and the range of detectable concentrations of antigen. It is known that real-time PCR gives exponential signal amplification and real-time detection. Under optimal conditions (100% efficiency) a 10-fold increase in the amount of DNA template is associated with a decrease in C_q value by a factor of 3.4. IPCR-based assays are therefore especially useful for detection of target antigens at large quantitative differences. In contrast, standard ELISA gives linear signal amplification and end point detection and, therefore, suits better for detection of smaller differences at lower range of concentrations; meaningful calibration curves for ELISA span usually two orders of magnitude or less.

Fifth, the labor requirement of the assays must also be taken into consideration. As shown in Fig. 1, Nano-iPCR based assays are less laborious because they have fever steps than iPCR or ELISA. Once the probes (functionalized Au-NPs) are prepared they can be stored for several months and used immediately for easy quantification of the antigen.

Our primary intention was to develop an assay for detection of cytokines in serum-supplemented cell culture media. Nano-

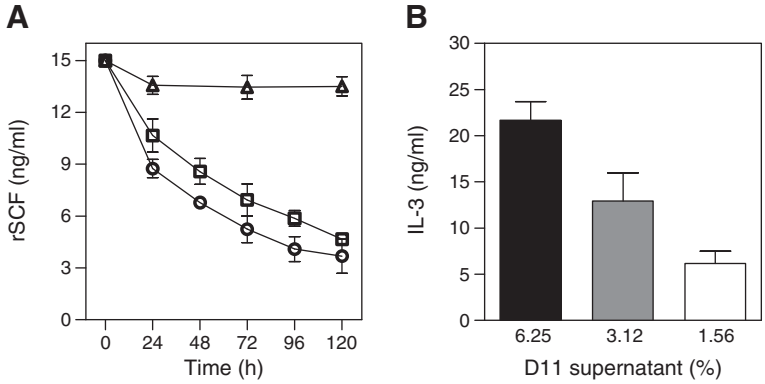


Fig. 6. Determination of SCF and IL-3 in culture media used for growth of BMMCs. (A) Changes in concentration of rSCF in culture medium with the length of time. The cells (1.0×10^6/ml) were cultured in complete RPMI-1640 medium supplemented with 15 ng/ml rSCF (0 h). Cell culture supernatants were collected at 24-h intervals for 120 h. The amount of rSCF was determined with Nano-iPCR I (circles) or ELISA (squares) using the corresponding calibration curves. As a positive control, supernatants from cell-free cultures were also analyzed in parallel by ELISA (triangles). (B) Quantification of IL-3 in supernatant from cultured D11 cells. Supernatant was diluted 16-fold, 32-fold or 64-fold in TPBS-1% BSA and the amount of IL-3 was determined with Nano-iPCR II using the corresponding calibration curves. Means and S.D. were calculated from 3 independent experiments.

Table 1
Comparison of the immunoassays used.

Parameter	Nano-iPCR	iPCR	ELISA
Reagents preparation	+	++	++
Tolerance for matrix	+	++	++
Sensitivity	+++	+++	++
Quantification range	+++	+++	+
Linearity	+++	+++	++
Variability	++	++	+++
Robustness	++	++	+++
Assay time	+++	+	++

+++, the best attribute; ++, the medium attribute; +, the worst attribute.

iPCR performed in TopYield strips with master mixes covered with oil film and transparent foil offers a simple and robust assay for rapid detection of IL-3 and SCF using commercially available antibodies and their biotinylated forms. The binding of antibody and thiolated oligonucleotide template to Au-NPs is an easy method of how to combine antibodies and oligonucleotides into a complex suitable for the assays. The assay can be used for quantification of other ligands, provided good monoclonal or polyclonal antibodies and their biotinylated forms are available.

In conclusion, Nano-iPCR assay shows enhanced sensitivity and wider dynamic range than ELISA and is easier to perform than iPCR. It can be expected that further improvement of the Nano-iPCR assays, namely introduction of better detection probes with higher ligand specificity and lower nonspecific binding will advance the application of these tests for routine detection of cytokines as well as other ligands. Synthetically prepared tailored DNA or RNA aptamers (Jayasena, 1999; Khati, 2010) and tailored recombinant binding proteins (Binz et al., 2005) could provide such probes.

Conflict of interest

The authors do not have a commercial or other association that might pose a conflict of interest.

Acknowledgments

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